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THE USE OF NEAMINE AS A MOLECULAR TEMPLATE: IDENTIFICATION OF ACTIVE SITE RESIDUES IN THE BACTERIAL ANTIBIOTIC RESISTANCE ENZYME AMINOGLYCOSIDE 3'-PHOSPHOTRANSFERASE TYPE IIa BY MASS SPECTROSCOPY

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Abstract: Four novel aminoglycoside-based affinity inactivators were shown to covalently modify the active site of aminoglycoside 3'-phosphotransferase type IIa (APH(3')-IIa), an important resistance factor in bacteria for aminoglycoside antibiotics. Standard peptide mapping techniques failed with this enzyme. A novel mass spectroscopic analysis which combines protease digestion on the instrument probe, followed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is described which permitted rapid identification of the sites of protein modification. By this new technique, Glu-3 and Asp-23 were identified as active-site residues, the side chains of which potentially may serve as counter ions for the ammonium functionalities at positions 6', and 1 and 3 of the antibiotic substrates, respectively. These findings contradict previous assertions that the C-terminal third of the enzyme should form the active site, by placing the active site clearly in the N-terminal portion of the enzyme.

Aminoglycoside 3'-phosphotransferase type IIa [APH(3')-IIa] is one of the most important resistance factors for aminoglycoside antibiotics. This enzyme catalyzes the transfer of phosphate from Mg^{2+}ATP to the 3'-hydroxyl of the antibiotics, and by so doing it inactivates the drug.[1-4] We described compounds **1-4** as molecules that underwent phosphorylation by the enzyme, so they were substrates. But, in the absence of Mg^{2+}ATP, these compounds inactivated the enzyme covalently by modifying nucleophilic amino acid residues in the active site.[5] This reaction is shown below for compound **3**. The bromoacetyl moiety would modify the nucleophilic residue closest to it on active site binding by the inactivator, as shown below for compound **4**. As such, these molecules have the potential of modifying different subsites of the active site, and allow for

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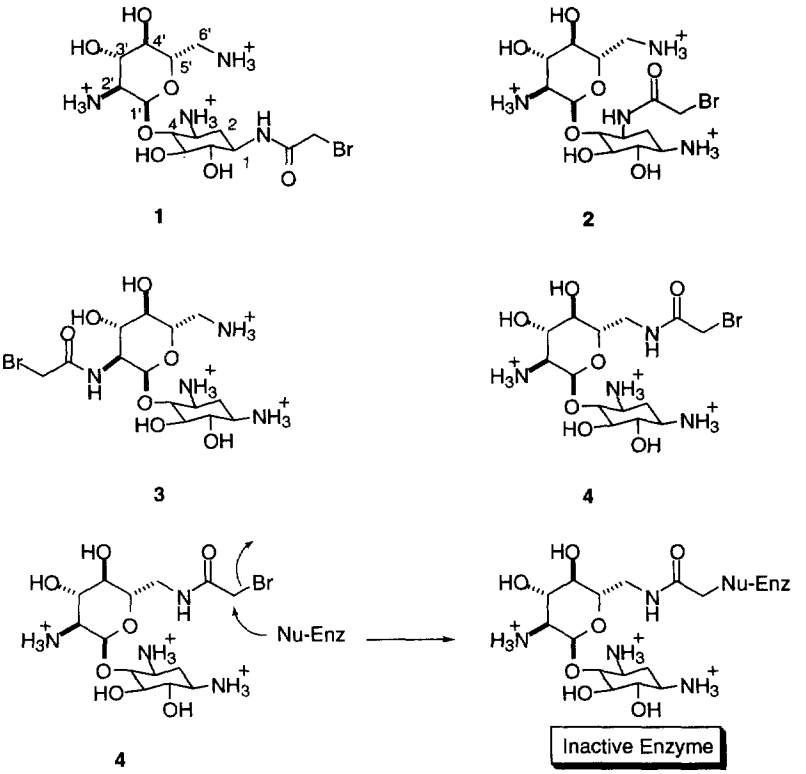


identification of different amino acid residues in the enzyme. Furthermore, we showed that the sites of modification were not the five cysteine residues found in this protein.[5]

Many attempts at standard methodology for fragmentation of the inactivated enzyme and peptide purification for sequencing failed. Therefore, we resorted to identification of the sites of protein modification by mass spectroscopy. The following description is for APH(3')-IIa modified by **4**, but the general procedure was applied similarly for the protein modified by each of the other inactivators. As shown in Figure 1, the enzyme was detected as the parent ion for both the modified and unmodified forms. Whereas the enzyme was predominantly modified by one inactivator, we also noted a small portion (estimated <15%) of enzyme modified by two inactivator molecules. The second modification could arise by a non-specific alkylation of the enzyme outside the active site. The presence of several small peaks with increasing mass increments of 39 corresponded to the formation of potassium adducts. The only assignment that could not be made was for the peak at M - 48.

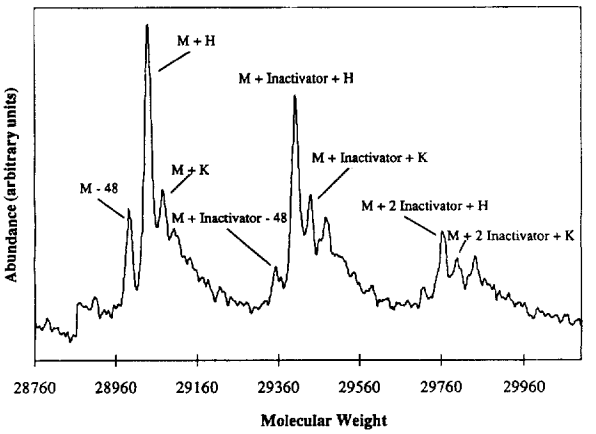


Figure 1. The reconstructed molecular weight plot obtained from ESI-MS analysis of APH(3')-IIa modified by compound **4**, and the relevant peak assignments. ESI-MS was performed on a PE Sciex API-III (Ontario, Canada)

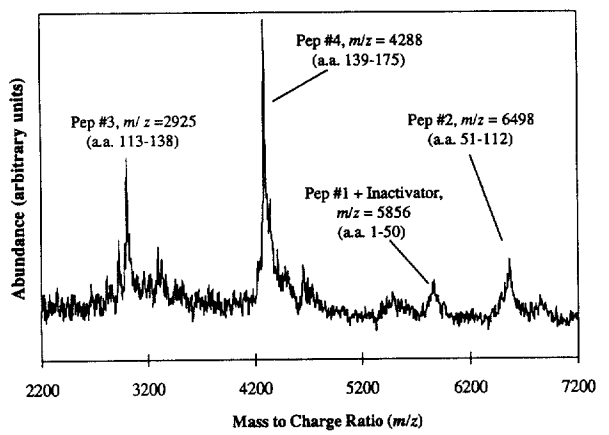


Figure 2. Mass spectrum of the mixture of peptides after complete hydrolysis by the endoprotease Lys-C of APH(3')-IIa modified by inactivator **4**. Peptide #5 does not appear in the *m/z* range indicated. MALDI mass spectra (for Fig. 2 and 3) were obtained on a Hewlett-Packard LDI-1700 XP (Palo Alto, CA, USA) MALDI mass spectrometer.

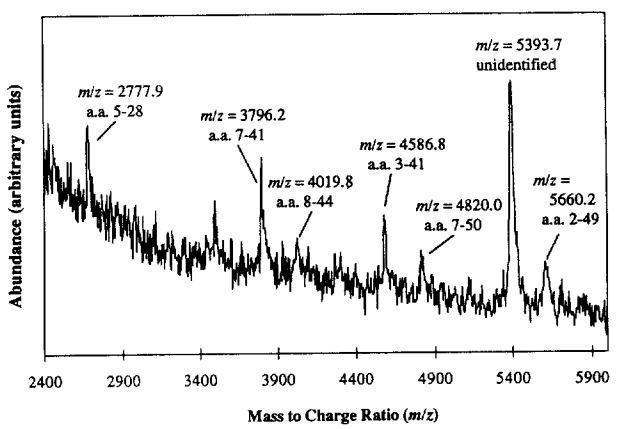


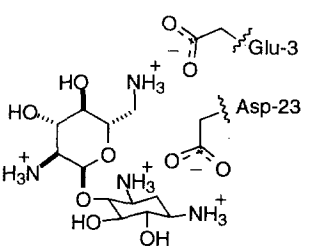
Figure 3. Mass spectrum of the mixture of partially hydrolyzed peptide comprised of residues 1-50 of the primary structure of APH(3')-IIa modified by inactivator **4**.

The modified enzyme was subsequently hydrolyzed by the endoprotease Lys-C. This protease hydrolyzes amide bonds where the carbonyl of the amide bond is provided by a lysine residue. Analysis of the mixture of peptides by matrix-assisted laser desorption ionization (MALDI) mass spectroscopy was performed on the mixture at this point. Based on the sequence of the amino acids for APH(3')-IIa, five peptide fragments of known sizes were expected for the products of complete hydrolysis of the enzyme by Lys-C, and that was seen. This analysis also identified the fragment that would have the inactivator appended to it. We were able to narrow down the site of modification by **4** to the peptide fragment comprised of amino-acid residues 1-50 (Figure 2). Subsequently, the peptide mixture from the Lys-C digest was purified by HPLC, and the modified peptide was identified by MALDI mass spectroscopy. The modified peptide was digested further by pronase for a brief time, which hydrolyzes the peptide backbone nondiscriminantly. This hydrolysis step was expected to produce a "ladder" set of incompletely digested peptides, which would be different from one another in size based on the random hydrolysis pattern. Subsequent to MALDI mass-spectroscopic analysis, several peptide fragments were identified (Figure 3). We could assign the sequences for these peptides (with the exception of the fragment with *m/z* of 5393.7), and also we determined which peptide contained the inactivator. The inactivator was associated with the peptides with *m/z* of 5660.2 and 4586.8, corresponding to amino-acid sequences spanning residues 2-49 and 3-41, respectively. All the remaining peptides, which spanned amino-acid sequences of 7-50, 8-44, 7-41 and 5-28, were unmodified. Hence, mass spectroscopy narrowed down the identity of the modified amino acid to either Glu-3 or Gln-4. Glutamate can serve as a nucleophile in the inactivation chemistry, whereas glutamine cannot. Hence, the site of protein inactivation by **4** is Glu-3.

The same type of analysis with APH(3')-IIa modified by **1**, **2** and **3** were performed. Regrettably, the results of our analysis for the enzyme modified by **3** did not reveal the site of protein modification. However, APH(3')-IIa modification by **1** or **2** was narrowed down to the peptide fragment spanning residues 1-50, as described for **4** already. Fragmentation of this peptide by pronase did not reveal the sites of protein modification. We assume that the pronase/MALDI treatment in the case of the enzyme modified by **4** was successful, as the site of modification was closer to the N-terminus. The peptide fragment was subsequently hydrolyzed by the endoprotease Asp-N, which shows preference for cleavage of amide bonds for which the nitrogen is provided by the amino-acid aspartate. This analysis limited the site of modification by either **1** or **2** to the span of the sequence of amino acids from residues 23-32. Within this portion of the sequence of APH(3')-IIa, only residues Asp-23 and Cys-31 have nucleophilic side chains. Our analysis of enzyme inactivation had revealed the cyanylated APH(3')-IIa experienced inactivation as did the native enzyme, arguing that Cys-31 should not be the site of modification.[5] To provide further support for this assertion, we carried out an additional mass spectroscopic experiment. We attempted to modify the fragment further by introducing an

acetamide moiety to the cysteine sulfhydryl group, should it be unmodified after inactivation of the enzyme by either **1** or **2**, and hence available for this reaction. Meanwhile, if the aminoglycoside inactivators were to be attached to Asp-23, the modification of the amino-acid side chain would generate an ester linkage that would be labile to basic conditions, a property that would not exist if the Cys-31 side chain was modified by the aminoglycoside inactivators. Hence, we mixed iodoacetamide and the peptide fragment under basic condition (pH 8.2), and then analyzed the nature of the peptide fragment. The result was quite clear, indicating that the acetamide moiety was incorporated into the peptide, and the aminoglycoside moiety was released (hydrolyzed) from it. Hence, this experiment confirmed that the site of protein modification with both inactivators **1** and **2** was Asp-23.

In this manuscript we have identified Glu-3 and Asp-23 as active-site residues for APH(3')-IIa. It was noted that the C-terminal third of the APH(3')s is rich in acidic residues, and it was suggested that this portion of the protein constitutes the active site,[6] in part based on the expectation that the formal charges of positive on the ammonium moieties of the substrates would be countered by the negatively charged side chains of such acidic residues. Furthermore, a stretch of eight amino acids at positions 204-211 in the primary structure of APH(3')-IIa aligns with portions of the sequences of several other phosphotransferases.[7] This C-terminal sequence was suggested to be involved in ATP binding.[7] Our results indicate that the aminoglycoside-binding portions of the active site of APH(3')-IIa are clearly in the N-terminal portion of the protein. Recently, Wright and colleagues have shown that residues Lys-33 and Lys-44 are modified in APH(3')-IIIa by an ATP-based affinity inactivator.[8] This finding also indicates that the N-terminal portions of the APH(3')s form part of the active site. We suggest that the side-chain carboxylates of amino-acid residues Glu-3 may be the counter ion to the substrate ammonium group at position 6', and that for Asp-23 should be near the ammonium groups at positions 1 and 3 of the substrates. This interaction is shown schematically below.



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